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SHORT COMMUNICATION

DNA barcoding of a stowaway reef coral in the international aquarium trade results in a new distribution record

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Abstract

Dead corals and limestone boulders that act as substrate for live specimens of marine invertebrates and algae are sold as 'live rock' in the international aquarium trade. During a customs inspection of an airfreight shipment of 'live rock' at Schiphol Airport (Netherlands), 450 boulders imported from Indonesia were checked for the presence of undeclared organisms. During unpacking, about 50% of the boulders appeared to have small stony corals attached to them. Some of these corals belonged to a species unknown from Indonesia. Mitochondrial COI and nuclear ITS markers revealed 100% and 99.3% match with *Polycyathus chaishanensis* Lin et al., 2012, a species reported from tidal pools in Taiwan. This new distribution record suggests that despite their easy access, intertidal and shallow subtidal reef coral assemblages (< 1 m depth) may still be underexplored.

Keywords CITES · Customs · Barcoding · COI · ITS · Geographical distribution · Indonesia · *Polycyathus* · Reef flat · Intertidal

Introduction

Tropical sea aquariums are important attractions in zoos around the world but are also kept at homes by numerous hobby aquarists. Consequently, there is a high global demand for ornamental reef fishes and invertebrates in the international aquarium trade (Dee et al. 2014). To mimic coral reef environments, large pieces of coral rock and limestone boulders are mined from coral reef areas and placed in aquariums as decoration and substrate (Padilla and Williams 2004). They are also known to clean sea water in aquaria (Yuen et al. 2009; Li et al. 2017). These boulders are most easily collected from shallow water and reef flats, which can be done by local villagers (Dawson Shepherd et al. 1992; Lovell 2001; Caras and Pasternak 2009). The boulders need to be inhabited and covered by live animals and plants from natural reef environments to serve as artificial reef habitats and are therefore called 'live rock' (Best 1997; Wood et al. 2012). These live organisms may belong to encrusting species of bryozoans, calcareous algae, sponges, zoantharians, corals, and other invertebrates that hide in crevices and borings or crawl over the rock surface, such as crustaceans, molluscs, echinoderms, and worms (Parks et al. 2003; Simões et al. 2017). According to CITES regulations (Convention on International Trade in Endangered Species of Wild Fauna and Flora), such attached specimens should not belong to coral species without proper export and import permits that mention their identity (Best 1997).

There is an extensive international trade in these 'live rocks'. They originally used to be exported from tropical countries like Fiji, Indonesia, and the Philippines, while the USA trade could get supplies from Florida and Hawaii (Bruckner 2001; Lovell 2001; Parks et al. 2003; Rhyne et al. 2009; Wood et al. 2012). In the last two decades, additional exporting countries have been recorded, such as Australia, Brazil, Cuba, Haiti, Palau, Samoa, Tonga, Vanuatu, and Vietnam, indicating that the harvesting of 'live rock' has been widespread due to an increasing economic demand (Table 1; Harriott 2001; Wabnitz et al. 2003;

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Gasparini et al. 2005; Rhyne et al. 2012; Gurjão and Lotufo 2018). Because organisms dwelling on 'live rock' are kept alive when they are transported over large distances, they can form a threat as potential invasive species in new areas (Padilla and Williams 2004; Bolton and Graham 2006; Walters et al. 2006; Morrisey et al. 2011) or as pest species in their new aquarium habitats (Rhyne et al. 2004; Calado and Narciso 2005).

To examine if such attached organisms belong to alien species, DNA barcoding (Hebert et al. 2003) can be applied for their identification (Wehr 2017; Vranken et al. 2018). This method represents a fast, simple, and economic tool to identify organisms based on DNA sequences and has been successfully used, for example, for the discovery of illegal trade and alien species of marine taxa (Collins et al. 2012; Bunholi et al. 2018). It can also be used to check whether imported organisms or products made from them, belong to species that are protected under CITES (Staats et al. 2016). The present study reports on the application of DNA-barcoding methodology to assist in the identification of scleractinian corals from Indonesia that were accidentally imported in the Netherlands.

Material and methods

During a customs inspection of an airfreight shipment of 'live rock' at Schiphol Airport (The Netherlands) on 22 October 2012, 450 imported coral boulders from Indonesia were checked for the presence of undeclared organisms (Fig. 1, ESM Figs. S1–S6). According to the accompanying documents, the shipment consisted of 80 boxes and had a weight of 4050 kg and a total value of USD 10,600 (including freight costs). The boulders (declared as "natural stone" and "substrate/unidentified scleractinians") were kept in moist condition inside styrofoam containers, which were packed in cardboard boxes. The packaging was done 2 days before arrival.

During unpacking, approximately 50% of the boulders appeared to have small scleractinian corals attached to them (Fig. 1). Some corals belonged to a species unknown from Indonesia, and one of these (Fig. 1b, c), with a colony diameter of 2.8 cm, was sampled and subjected to DNA barcoding for species identification. In detail, DNA extraction was carried out in the molecular laboratory of Naturalis with the DNeasy Blood and Tissue kit (Qiagen Inc., Hilden, Germany). Two loci were selected for DNA barcoding, a portion of the mitochondrial cytochrome *c* oxidase subunit I (COI, partially) and a selection of nuclear rDNA (ITS, including the entire ITS1, 5.8S, ITS2, and a fragment of 18S and 28S). COI was amplified using primers fungCOIfor1 (5′-CTG CTC TTA GTA TGC TTG TA-3′) and fungCOIrev2 (5′-TTG CAC CCG CTA ATA CAG-3′) (Gittenberger et al. 2011), and ITS using primers ITS4 (5′-TCC TCC GCT TAT TGA TAT GC-3′) (White et al. 1990) and A18S (5′-GAT CGA ACG GTT TAG TGA GG-3′) (Takabayashi et al. 1998). Amplifications were performed in a 12.5-mL PCR reaction mix containing 0.2 mM of each primer, 2 X Multiplex PCR Master Mix (Qiagen Inc., Hilden, Germany) and < 0.1 ng of DNA. The thermal cycler profile was of 95 °C for 15 min, 30 cycles of 95 °C for 1 min, 53 °C for 1 min (50 °C for ITS), 72 °C for 1 min, with a final phase of 72 °C for 5 min. PCR products were directly sequenced in forward and reverse directions using an automated 3730xl DNA Analyzer (Applied Biosystem, Foster City, CA, USA). Newly obtained COI and ITS sequences from the analysed specimen were checked via Blast searches against nr GenBank database. Subsequently, they were aligned with closely related coral sequences available in GenBank using MAFFT (Katoh and Standley 2013) and the iterative refinement method E-INS-i. The phylogenetic trees were obtained using maximum likelihood criterion using RAxML (Stamatakis 2014) with a multiparametric bootstrap analysis of 500 bootstrap replicates. Newly obtained sequences of COI and ITS were deposited in NCBI under the accession numbers MN533979 and MN527240, respectively.

To keep the specimen available for future research, it has been deposited in the scientific reference collection of Naturalis Biodiversity Centre (Leiden, the Netherlands) with catalogue number RMNH.COEL.42435).

Results and discussion

Most corals attached to the imported 'live rock' could easily be identified to genus or species level, such as *Coeloseris mayeri* Vaughan, 1918 and *Pavona* sp. (both Agariciidae), *Cyphastrea* sp. and *Merulina* sp. (both Merulinidae), *Montipora* sp. (Acroporidae), *Psammocora* sp. (Psammocoridae), and *Porites* sp. and *Stylaraea punctata* (Linnaeus, 1758), both Poritidae. *Stylaraea punctata* is typical for shallow, intertidal areas (Kitano et al. 2014; Richards et al. 2015), and widespread in Indonesia (Hoeksema 2004).

One species in the investigated shipment was unknown for Indonesia and needed further analysis for its identification. The soft tissue of this coral showed a brown colour (Fig. 1b–d), suggesting that it is zooxanthellate. The thecae of the tubular calices were almost completely covered by encrusting coralline algae (Fig. 1d). Its corallum shape agreed with that of *Polycyathus* Duncan, 1876 (Caryophylliidae), a genus of 19 recognized species worldwide (Hoeksema and Cairns 2018). Many of these species are exclusively known from deep water, and therefore, all of them are usually considered as azooxanthellate corals (e.g., Cairns et al. 1999; Cairns and Kitahara 2012).

COI and ITS markers revealed a 100% and 99.3% match with specimens of *Polycyathus chaishanensis* Lin et al., 2012 (ESM Figs. S7–S8) collected from its type locality in Taiwan (Lin et al. 2011, 2012). So far, this species has only been recorded from here and two other localities in Taiwan, each

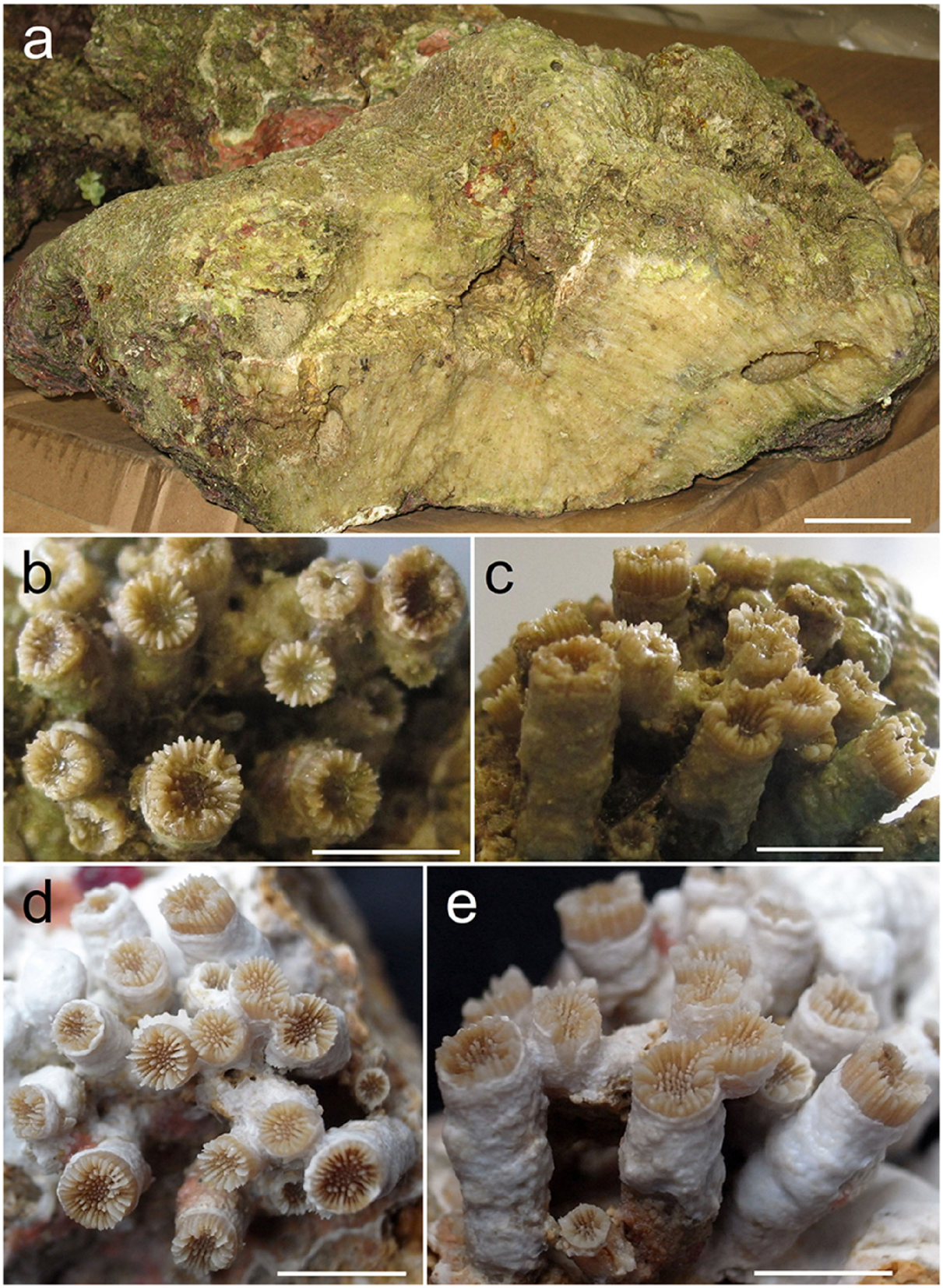

Fig. 1 **a** Dead coral boulder shipped as 'live rock', subject to customs inspection at Schiphol Airport (scale bar: 5 cm); **b**, **c** Live specimen of *Polycyathus chaishanensis*: polyps seen from above (**b**) and aside (**c**); **d**, **e** Skeleton of the same specimen after cleaning in household bleach; polyps seen from above (**d**) and aside (**e**); catalogue nr. RMNH.COEL.42435. Scale bars **b**–**d**, 5 mm

time in shallow water from intertidal to 3 m depth (Kuo et al. 2019). The phylogenetic analyses based on both COI and ITS loci showed that our newly obtained sequences clustered together with sequences of *P. chaishanensis* from Taiwan with high bootstrap values (100 for both analyses). Moreover, the three sequences of *P. chaishanensis* from Taiwan formed a single lineage with low-moderate bootstrap value (63) in the ITS tree. Therefore, the high value of genetic identity based on Blast searches and the results from the phylogeny reconstructions seem to suggest that our specimen can be identified as *P. chaishanensis* based on the few molecular data that are actually available. Nevertheless, since molecular information on other *Polycyathus* species has not yet been published, we suggest the inclusion of more sequences of shallow-water species ascribed to this genus from different localities to get a more complete geographic and taxonomic sampling of this genus for future genetic analyses.

Morphological characters were also studied. The specimen from Indonesia showed calices that are 2.5–3.5 mm wide, with 24–32 septa divided over three to four cycli and a length of 1 cm (Fig. 1b–e). According to its original description, *P. chaishanensis* shows a maximum calice diameter of 3.7 mm and up to 34 septa in up to four cycli (Lin et al. 2012). Morphologically, it resembles *P. hodgsoni* Verheij and Best, 1987, which has been recorded from caves at depths over 35 m in the Philippines and the Maldives (Verheij and Best 1987). This species has smaller and shorter calices (2–3 mm in diameter, < 5 mm long) with 20–24 septa divided over three cycli. Other *Polycyathus* species reported from the central Indo-Pacific have calices that are over 5 mm in diameter: *P. fulvus* Wijsman-Best, 1970, known from very shallow water (0.3–0.5 m depth) in New Caledonia (Wijsman-Best 1970); *P. isabela* Wells, 1982 from 14 m depth at Galápagos (Wells 1982); *P. furanaenis* Verheij & Best, 1987 found in caves at 6–52 m depth (Verheij and Best 1987); *P. marigondoni* Verheij & Best, 1987, which has been found in a cave at 35 m depth (Verheij and Best 1987); *P. andamanensis* Alcock, 1893, known from dredges at unknown depths; and *P. octoplus* Cairns, 1999, dredged from 110 to 441 m depth. With regard to the environment, only *P. fulvus* with larger polyps appears to share a similar habitat with *P. chaishanensis*. To obtain a better overview of *Polycyathus* species, it appears that this genus is in need of a worldwide taxonomic revision, in which molecular, morphological, biogeographic, and bathymetric characteristics are taken into account. It is also important to photograph fresh specimens or examine specimens stored in ethanol to verify whether zooxanthellae are present or not, because for collected specimens, it is usually assumed that they are azooxanthellate, as in the case of *Polycyathus fuscomarginatus* (Klunzinger, 1879) trawled from 10 to 30 m depth off Chennai, Southern India (Venkataraman 2007).

The question arises whether more distribution records of rarely known coral species can be obtained from customs inspections. Large quantities of 'live rock' (mostly from Indonesia) are imported in the Netherlands (Table 1), whereas the total export of 'live rock' from Indonesia to other countries in 2012 (the year of the present example) was almost 10^6 kg (Table 2). How frequently shipments of 'live rock' are being inspected by customs officers is not known. Since the imported rocks only need to be inspected for the presence of CITES-listed species at high taxonomic level (mostly Scleractinia spp.), assistance given by a coral taxonomist may not be necessary in this case. On the other hand, the present study can serve as a test case for illustrating the usefulness of DNA barcoding in the identification of protected species or their products, like previously applied to cacti (Gathier et al. 2013), medicinal plants (Eurlings et al. 2013), sharks (Liu et al. 2013; Feitosa et al. 2018), orchids (de Boer et al. 2017), and trees (Yu et al. 2017). Therefore, knowledge on the trafficking of rarely known species in the aquarium trade may benefit from more inspections assisted by expert taxonomists who can select the specimens to be used for DNA barcoding.

It is indeed remarkable that *P. chaishanensis* has not been reported from other localities despite its occurrence in shallow tidal pools and reef flats, as reported from Taiwan (Lin et al. 2012; Kuo et al. 2019). This scarcity of records suggests that shallow reef habitats in the tropics, despite their easy access, could be understudied and may house more infrequently observed species. Because most coral researchers use SCUBA equipment at depths over 3 m, they may overlook coral species occurring in intertidal and shallow subtidal habitats (< 1 m depth), such as *Stylaraea punctata* (Linnaeus, 1758) at Guam, which can easily be found in tidal pools on reef flats during low tide (Randall and Myers 1983; Golbuu and Richmond 2007; BWH pers. obs.). Another example is the free-living morphotype of the scleractinian coral *Favia gravida* Verrill, 1868, which is only known from rocky tide pools of Ascension Island (Hoeksema 2012; Hoeksema

Table 1 Import of 'live rock' (kg) in the Netherlands from various countries over the years 2009–2018 (CITES 2020)

Year	Import (kg)				
	Cuba	Fiji	Indonesia	Vanuatu	Vietnam
2009		2800	15,000		
2010		2600	5500		
2011	1000	2800	23,100		
2012	3000	3100	38,300		
2013	16,500	19,000	35,700		
2014	3800	5900	46,800		13,700
2015		3500	38,900		300
2016		35,000	33,300	16,000	200
2017		300	32,600	13,800	600
2018	14,000		10,200		

Table 2 Import of 'live rock' in various countries from Indonesia in 2012; * = based on export data from Indonesia (CITES 2020)

Year	Import (kg)
Austria	7300
Belgium	8200
Canada*	53,900
Czechia	1200
Denmark	1600
Finland	1700
France	66,900
Germany	101,900
Greece*	2600
Honk Kong	3200
Israel*	3000
Italy	18,400
Japan	73,000
Korea	19,000
Mexico	3800
Netherlands	38,300
New Zealand	700
Norway	1000
Poland	15,100
Portugal	800
Russia*	26,500
Saudi Arabia*	300
Spain	8200
South Africa	1000
Sweden*	12,000
Switzerland	3000
Taiwan*	3200
United Arabian Emirates	11,900
UK	78,900
USA	357,300
Total	**923,900**

and Wirtz 2013). These observations suggest that shallow reef environments can harbour unique coral reef fauna and that future surveys here may result in new species records.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval No animal testing was performed during this study.

Sampling and field studies All necessary permits for sampling were obtained by the authors from relevant authorities and are mentioned in the acknowledgements when applicable.

Authors' contribution BWH collected the examined specimen at Schiphol Airport. Both authors wrote the manuscript. BWH studied morphological characters. AR performed the molecular analysis. Both authors read and approved the manuscript.

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